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Full Length Research Paper

Evaluation of nanosilver efficacy along with transforming growth factor (TGFβ) in induction of chondrogenesis: An animal study

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This study is on the multipotential mesenchymal cells which enable us to differentiate chondroblasts in presence of Transforming growth factor (TGFβ) and bone morphogenic factors (BMPs). As use of a transferring system for these growth factors is a notable issue in recent researches, the present study was designed to evaluate the nanosilver transferring system of TGFβ on two sample groups including 42 rats and 4 rabbits as pilot study. The samples were divided in two groups for injection of TGFβ with and without nanosilver. Femoral muscle of rats were injected and then sacrificed after 1, 2 and 4 weeks. This injection was repeated in subperiosteal area of parietal bone of rabbits with decreased dose of nanosilver. The injected areas were examined histopathlogically using H&E and Methylene-blue staining methods. Results revealed chondrogenesis was not detected in all 6 rat groups in all following weeks, but was present in the parietal bone of one rabbit which was injected with TGFβ with nanosilver and sacrificed after 4 weeks. The differences in level of inflammation in and presence of necrosis were significant in TGFβ with nanosilver groups vs TGF ones. Although, addition of nanosilver to TGFβ did not show great cytotoxic responses, it may not have great effects to induction of chondrogenesis in rat femoral muscle. Detection of chondrogenesis in one sample of subperiosteal rabbit parietal bone can be explained by decreased nanosilver dose and difference in anatomic site of injection. So, more investigations need to verify practical use of TGFβ with nanosilver transferring system in repair of chondral defects.

Key words: Transforming growth factor (TGFβ), bone morphogenic factor (BMP2), nanosilver, chondrogenesis.

INTRODUCTION

Repair of chondral defects is a notable issue after bony defects in the recent oral and maxillofacial surgery progressions (Mow et al., 1992). The chondral tissue has a good physical strength and also play great role in development and stability of facial skeleton; however, there is a low reconstruction potential after traumatic injuries due to absence of vascularity (Guo et al, 1989). Some clinical considerations were targeted towards reconstruction of chondral defects such as autografts and subchondral drilling; however these are accompanied by some technical failures such as donor site morbidity, few number of chondroblasts in graft tissue, progression to articular osteoartheritis, fibrosis and local infection (Caplan and Goldberg, 1999; Buckwalter and Mankin, 1998; Messner and Gillquist, 1996; Brittberg, 1996; Grigolo et al., 2005).

Many researches were found in recognition of contributing factors in *in vitro* induction of chondrogenesis. Also, use of undifferentiated

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Table 1. Level of inflammation in rat sample groups in following weeks.

Injected material/Time	First week	Second week	Fourth week
TGFβ	1± 0	1± 0	0
TGFβ+nanosilver	2.71± 0.49	1.43± 0.53	1± 0

mesenchymal cells would be a notable issue in reconstruction of chondral defects in these researches (Caplan and Goldberg, 1999; Brittberg, 1996; Barry et al., 2001). The undifferentiated mesenchymal cells can be differentiated from chondroblasts in presence of growth factors as Insulin-like growth factor (IGF), Transforming growth factor (TGF) and Bone morphogenic factor (BMP) (Erickson et al., 2002; Sheyn et al., 2008).

The higher dose of these factors or combination of them was recommended to achieve this differentiation (Hennig et al., 2007; Kim and Im, 2008; Kim and Im, 2009). These growth factors have sensitivity to increased pH level, temperature and proteolysis procedures. So, use of a transfering system is mandatory for precise transport of the growth factors and also increased their bioactivity (Silva et al., 2009; Sung et al., 2010).

Nano particles enable the penetration of biologic barrier due to minute size of them, so they can affect the cellular physiology. Indeed, they are used for increasing intracellular entrance of therapeutic agents (Laura et al., 2005; Chen et al., 2003; Chavany et al., 2003). These particles were employed as *in vitro* carrier of growth factors but there were no reports around *in vivo* use of them (Sung et al., 2010; Laura et al., 2005; Chen et al., 2003; Chavany et al., 2003; Richardson et al., 2001).

Nanosilver particles have been used as antibacterial agent for wound healing procedures and also in bone cement ingredients (Chu et al., 1988; Wyatt et al., 1990; Volker et al., 2004). It has no significant efficacy on mesenchymal cells, fibroblasts and osteoblasts in low concentration although it would be bactericidal for *Staphylococcus aureus* and some fungal species (Laura et al., 2005; Chen et al., 2003; Chavany et al., 2003; Richardson et al., 2001; Chu et al., 1988; Wyatt et al., 1990; Volker et al., 2004; Michel et al., 1990; Sakaguchi et al., 2005; Lieberman et al., 2008; Jaklenec et al., 2008; Dandan et al., 2007). So, the present study was designed for evaluation of TGFβ with or without nanosilver particles as a transfer to induction of chondrogenesis in rat femoral muscle and rabbit subperiosteal parietal bone.

MATERIALS AND METHODS

42 male Sprague Dawley rats with 200 to 220 g weight were obtained and divided equally into 6 groups. Colloidal nanosilver solution was purchase from Germany Plasmachem Company. The 200 ngr TGFβ which was purchased from Korean Company of Biomed Hans, solved in 0.4 cc normal saline with pH=7.4, then injected into rat femoral muscle of groups 1, 2 and 3 with 25 gauge needle.

The 200 ngr TGFβ was mixed with 0.1 cc of colloidal nanosilver by concentration of 0.1 mg/cc which contained 10 µg nanosilver and solved in 0.5 cc normal saline. Then, the mixture was injected into the mentioned muscle of groups 4, 5 and 6. The normal saline with same acidity was injected as a control into opposite femoral muscle in all groups.

Groups 1 and 4 were sacrificed after 1 week, Groups 2 and 5 after 2 weeks and the rest after 4 weeks with Thiopental-Na overdose method (Michel et al., 1990).

The injection area was prepared for histopathological examination, using H&E method; necrosis, inflammation were evaluated by a pathologist with optic microscope (ZIEZZ, Germany) by ×200 and ×400 magnification. Level of inflammation was evaluated as 4 scores: 0 for absence of it, 1 for mild, 2 for moderate and 3 for severe inflammation (Anna and Fuks, 1997). Presence of necrosis was also evaluated in this step. Induction of chondrogenesis was examined and suspected samples were stained by methylene-blue staining method.

In the next step, 5 µg nanosilver particles was solved in 0.1 cc colloidal solution; then injected along with 200 ngr TGFβ into subperiosteal parietal bone of 2 white male rabbits. Two other rabbits were injected with 200 ngr TGFβ without nanosilver particles. The samples were sacrificed by same method of rats and injection areas were prepared for histopathological examination.

RESULTS

The result of evaluation in 42 rats and 4 rabbits in two groups of TGFβ and TGFβ with nanosilver particles in following periods of 1,2 and 4 weeks is described here. None of the six rats groups show chondrogenesis in all following periods. Histopathological examination of the injection areas revealed foreign body material and foci of inflammatory foreign body reaction including inflammatory cells, giant cells, necrosis and abscess formation with no evidence of chondrogenesis (Figure 1). Level of inflammation in rat samples according to their following weeks has been shown in Table 1.

In the first week, this level was significantly higher in nanosilver with TGFβ groups vs TGFβ ones (control group) ($p<0.05$). In addition, inflammatory cells and areas of abscess formation were detected in 4 of 7 samples in nanosilver with TGFβ groups in first week (Figure 2) which were not found in control groups.

Surprisingly, one of the rabbits which was injected by TGFβ with nanosilver and sacrificed after 4 weeks demonstrated areas of chondrogenesis (Figures 3 and 4).

Also, these areas were stained by methylene-blue for specific evaluation of chondrogenesis and fortunately,

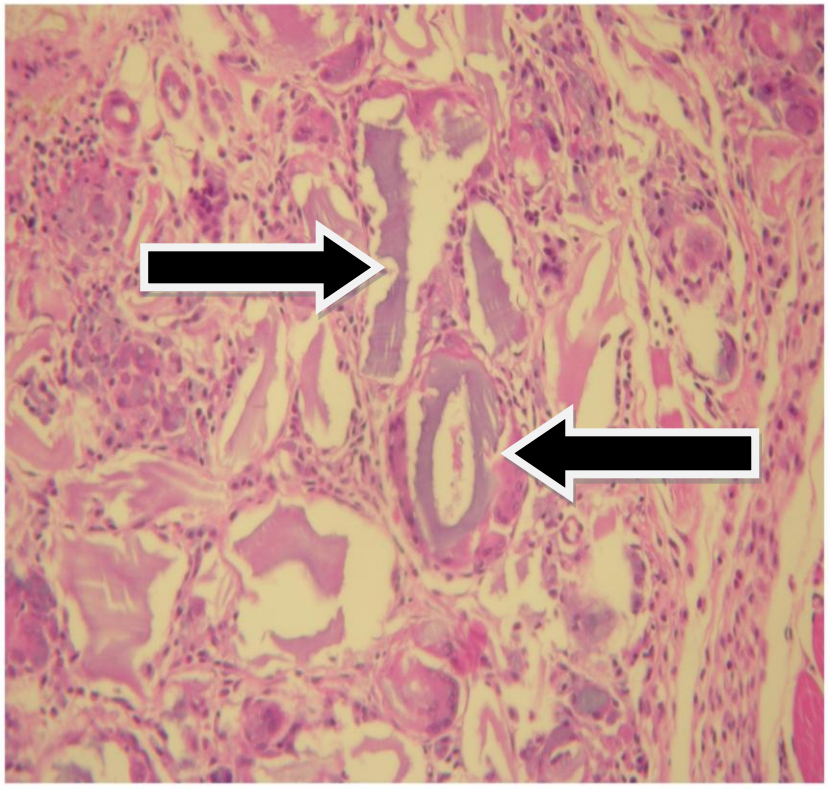

Figure 1. Histopathologic examination of the injection areas showing foreign body material (arrow pointed) and foci of inflammatory foreign body reaction including inflammatory cells with no evidence of chondrogenesis (×200 magnification).

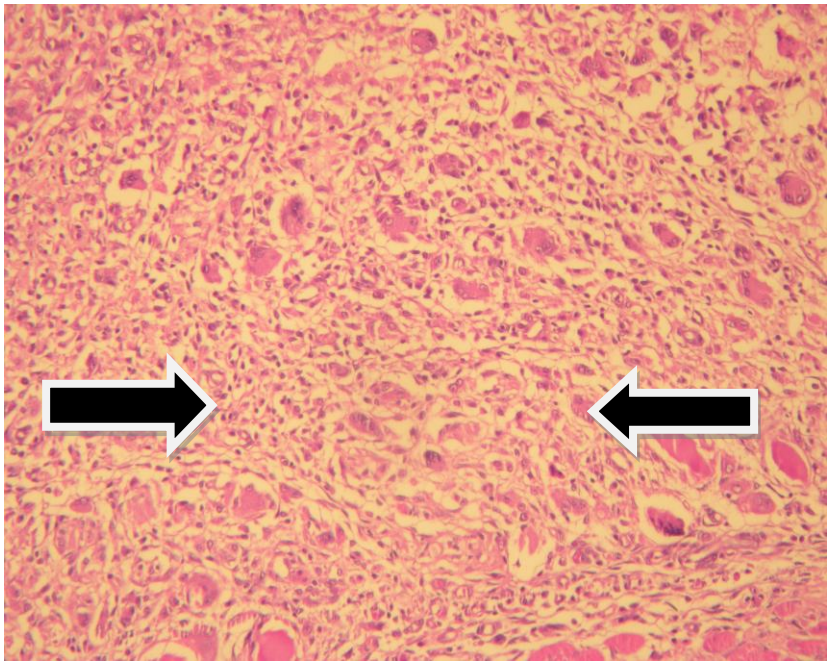

Figure 2. Area of abscess formation containing inflammatory cells and numerous giant cells (arrow pointed) in 4 of 7 samples in nanosilver with TGFβ groups in first week.

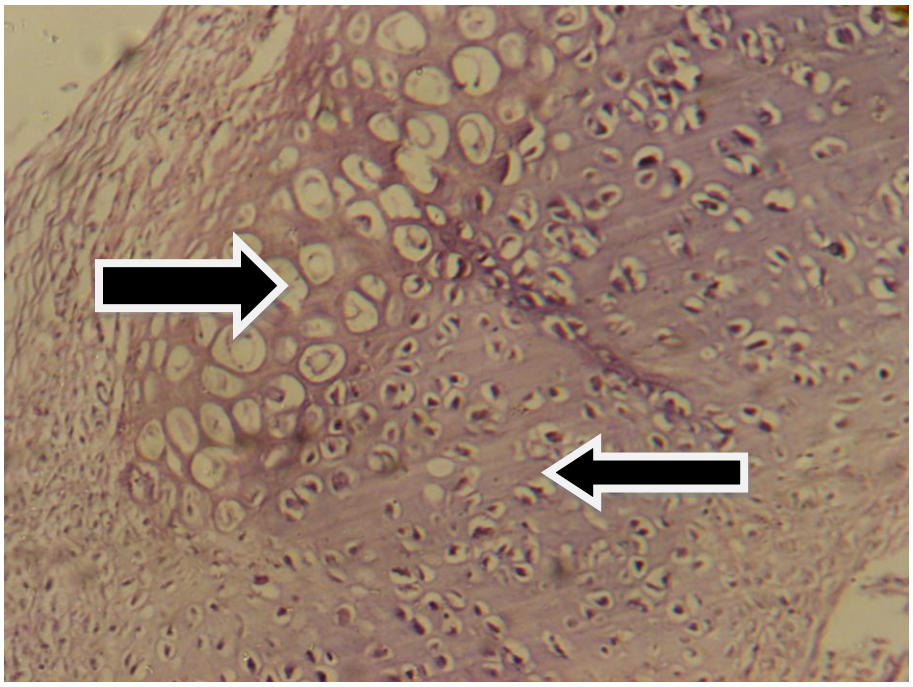

Figure 3. H&E staining of subperiosteal area of rabbit parietal bone shows chondroblastic differentiation pointed by arrow (x400 magnification).

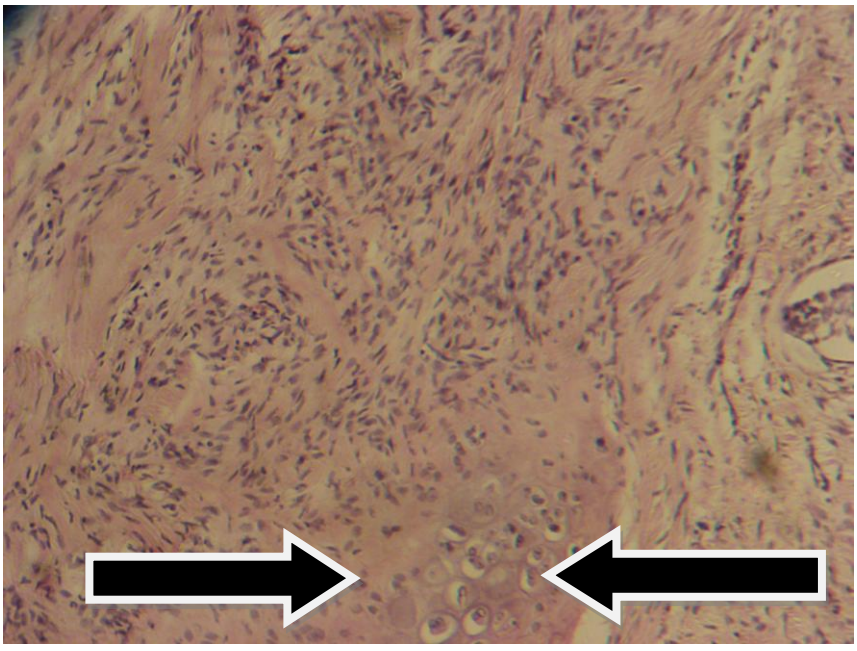

Figure 4. H&E staining of subperiosteal area of rabbit parietal bone (arrow shows differentiation of chondroblasts, cut section is on 90° related to Figure 1 (×200 magnification).

staining were positive in these sections (Figure 5).

In cases of negative controls, which were injected by normal saline no obvious histopathological changes were seen.

Presence or absences of necrosis in rat samples were shown in Table 2 in following weeks. In the first week,

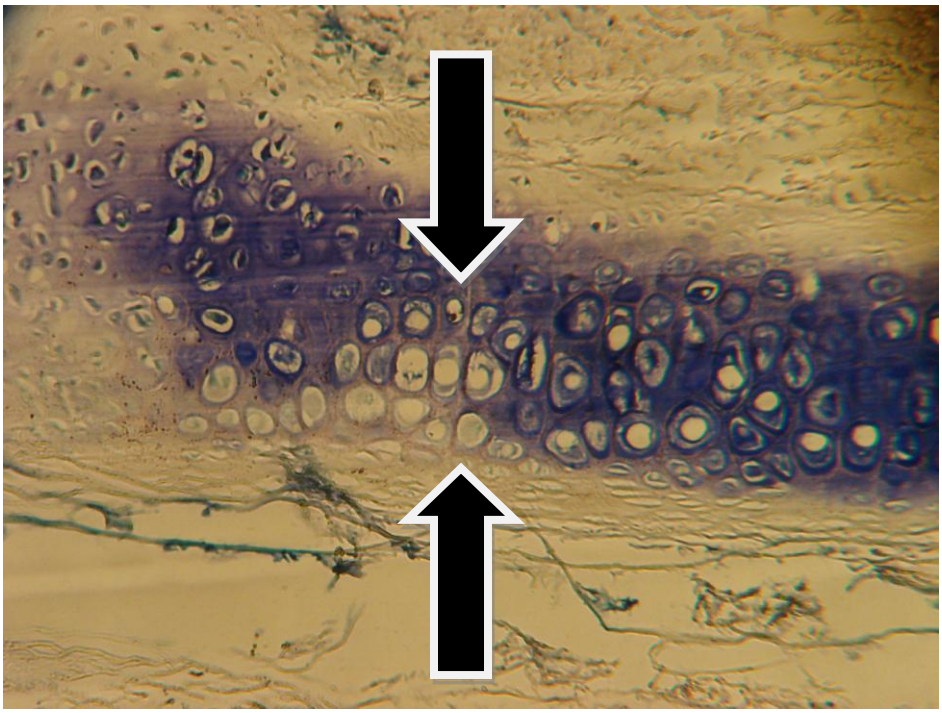

Figure 5. Methylene-blue staining of section considered to be chondrogenesis. Areas of blue staining (arrow pointed) shows chondroblastic differentiation (×400 magnification).

Table 2. Presence of necrosis in rat sample group in following weeks.

Factors	First week		Second week		Fourth week	
Necrosis	yes	no	yes	no	yes	no
TGFβ	7	0	7	0	7	0
TGFβ+ nanosilver	3	4	7	0	7	0

there was no evidence of necrosis in TGFβ groups but some areas of necrosis were present in TGFβ with nanosilver groups. This difference was statistically significant ($p<0.04$), however these areas disappeared in the following weeks in all sample groups.

The result of inflammation levels and presence of necrosis were evaluated statistically by Man- U Whitney and Fisher exact tests, respectively and $p<0.05$ was considered significant.

DISCUSSION

The undifferentiated multipotential mesenchymal cells were the great source of reserve cells for reconstruction of chondral defects (Volker et al., 2004; Michel et al., 1990; Sakaguchi et al., 2005). Bioactive molecules such as growth factors and cytokines have an important role during differentiation to chondroblasts as proliferation and differentiation of these mesenchymal cells. During this differentiation, presence of high dose growth factors like IGF, TGFβ and BMP would be mandatory, but there is an *in vitro* challenging point here due to stability and activity of high dose growth factors (Lieberman et al., 2008).

To approach this challenge, some researches were designed to co-incidentally use some growth factors to intensify their synergic effects and others experienced a transferring system of nanoparticles for these factors. This system was believed to increase stability and bioactivity of growth factors (Kim and Im, 2008; Jaklenec et al., 2008).

The possible explanation is changing of ionic specifities by these particles which inhibited collection, agglutination and precipitation of growth factors. So, it would result in increasing of stability, bioactivity, controlled releasing of these factors and also long lasting presence of multipotential mesenchymal cells. The recent point can result in increased induction of these cells to chondroblasts (Sung et al., 2010). On the other hand, nanosilver particles presented antibacterial effects, too;

but there is no evidence of toxicity on human osteoblasts and undifferentiated cells by concentration of 10 µgr and lower (Volker et al., 2004). In the present *in vivo* study, the animal samples were used for evaluation of nanosilver particles as transferring of growth factor of TGFβ to differentiated multipotential cells to chondroblasts. The subperiosteal areas are believed to be a great source of these cells (Michel et al., 1990).

TGFβ with and without nanosilver particle were injected into the femoral muscle of rats and subperiosteal parietal bone of rabbits. They were sacrificed after 1, 2 and 4 weeks and evaluated histopathologically for induction of chondrogenesis, inflammation and necrosis. There were no evidence of chondrogenesis in all rat groups but it was obvious in one of the rabbits which were injected by 200 ngr TGFβ with 0.1 cc colloidal solution containing 5 µgr nanosilver.

Michel et al. (1990) injected 200 ngr of TGFβ to femoral muscle for consequent days. They reported differentiation of chondroblasts in fourth day.

As the present study was designed to evaluate nanosilver, we did not follow the consequent approach for injection. Indeed, absence of chondrogenesis was predictable in control groups; although for sample groups this issue can be referred to as ineffectiveness of nanosilver particles to stability and bioactivity of growth factor in injected area which can lead to differentiation of mesenchymal cells to chondroblasts. In addition, presence of necrosis and inflammation may be related to high dose of nanosilver particles which was more obvious in sample group vs control in the first following week.

Danden et al. (2007) implanted solid nanosilver in the rat femoral muscle which show good biocompatibility during 30 days following period; however, it caused cyst formation and inflammation after 30 days.

In the present study no evidence of cyst formation was reported and only inflammatory cells were detected. Furthermore, level of inflammation was decreased in second and fourth week's vs first week. It seems that this referred to low concentration of nanosilver and use of colloidal type of it which inhibited the agglutination of particles.

To evaluate dose related results, nanosilver dose was decreased for rabbit group (5 µg in 0.1 cc colloidal solution vs 10 µ) and injected along with 200 ngr TGFβ to subperiosteal parietal bone of rabbits. Histopathological examination of injection area revealed induction of chondrogenesis in one rabbit of the sample group after following four weeks.

So, it seems that absence of chondrogenesis in rat samples referred to high dose of injected nanosilver particles which caused inflammation and increasing of blood perfusion and systemic absorption of growth factors. Also, this increased dose caused a deregulation in peripheral equivalency and inhibited better performance of growth factors.

Furthermore, level of inflammation was lower in rabbit group vs rat group in following four weeks which referred to decreased dose of nanosilver and its host response to it. In addition, it seems that, presence of a suitable anatomic barrier would be useful in induction of chondrogenesis. The possible explanation is: the growth factors were absorbed systemically faster in muscle tissue which has a more blood perfusion, so they were omitted faster and would have a little chance to induction of chondrogenesis.

Conclusion

It seems that, addition of nanosilver to TGFβ have no great effect in induction of chondrogenesis in rat femoral muscles; although it would be present in one sample of subperiosteal rabbit parietal bone which can be probably explained by decreased nanosilver dose and difference in anatomic site of injection or spices of animals. In addition, nanosilver particles revealed few inflammatory responses which were found to have disappeared after some weeks. According to present studies we can suggest that, in the subperiosteal areas which have slow systemic absorption of growth factors, more effective differentiation of undifferentiated cells to chondroblasts were occurred but more investigations need to be done for practical use of TGFβ with nanosilver transferring system in repair of chondral defects.

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